Biochimica et Biophysica Acta 1812 (2011) 752–759

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Systemic administration of high-molecular weight hyaluronan stimulates wound healing in genetically diabetic mice

Mariarosaria Galeano [a,b,1], Francesca Polito [a,c,1], Alessandra Bitto [a,1], Natasha Irrera [a], Giuseppe M. Campo [c], Angela Avenoso [c], Margherita Calò [d], Patrizia Lo Cascio [e], Letteria Minutoli [a], Mauro Barone [f], Francesco Squadrito [a,*], Domenica Altavilla [a]

[a] Department of Clinical and Experimental Medicine and Pharmacology, Section of Pharmacology, School of Medicine, University of Messina, Italy
[b] Department of Surgical Sciences, Section of Plastic Surgery, School of Medicine, University of Messina, Italy
[c] Department of Biochemical, Physiological and Nutritional Sciences, School of Medicine, University of Messina, Italy
[d] Department of Experimental Sciences and Applied Biotechnology, School of Veterinary Medicine, University of Messina, Italy
[e] Department of Food and Environmental Sciences, University of Messina, Italy
[f] Campus Bio-Medico, Rome University, Rome, Italy

ARTICLE INFO

Article history:
Received 26 November 2010
Received in revised form 15 February 2011
Accepted 21 March 2011
Available online 3 April 2011

Keywords:
Hyaluronic acid
Diabetic mice
Cyclins
Transglutaminase-II

ABSTRACT

Hyaluronic acid (HA), an essential component of the extracellular matrix, is an efficient space filler that maintains hydration, serves as a substrate for assembly of proteoglycans and is involved in wound healing. Although numerous pieces of evidence demonstrate beneficial effects in promoting wound healing in diabetes, a systemic approach has never been tested. We used an incisional wound healing model in genetically diabetic mice to test the effects of systemic injection of HA. Diabetic ($n=56$) and normoglycemic ($n=56$) mice were subjected to incision and randomized (8 groups of 7 animals each) to receive HA at different doses, 7.5, 15 and 30 mg/kg/i.p., or vehicle (0.9% NaCl solution) for 12 days. At the end of the experiment animals were sacrificed and skin wounds were excised for histological, biochemical and molecular analysis. Histology revealed that the most effective dose to improve wound repair and angiogenesis in diabetic mice was 30 mg/kg. Furthermore HA injection (30 mg/kg) improved the altered healing pattern in diabetic animals, increased skin remodeling proteins TGF-β and transglutaminase-II and restored the altered expression of cyclin B1/Cdc2 complex. Evaluation of skin from diabetic animals injected with HA revealed also an increase in HA content, suggesting that systemic injection may be able to restore the reduced intracellular HA pool of diabetic mice. Finally HA markedly improved skin mechanical properties. These promising results, if confirmed in a clinical setting, may improve the care and management of diabetic patients.

1. Introduction

Diabetic patients have impaired wound healing associated with increased morbidity and mortality [1,2]. The majority of non-healing wounds often leads to amputation, thus increasing the direct costs of their care, rehabilitation, and lost productivity [3]. Clinical and experimental evidence suggests that diabetic ulcers and other types of chronic wounds do not follow an orderly and reliable progression of [1].

Hyaluronic acid (HA) consists of a basic unit of two sugars, glucuronic acid and N-acetylglucosamine, polymerised into large macromolecules of over 30,000 repeating units. It is therefore one of the largest components of the extracellular matrix and its structure, highly conserved in evolution, appears identical in rodents and humans [4]. The molecule is readily soluble in water, producing a gel and has no allergenic properties, representing an excellent molecule for clinical applications. HA is formed at the cell surface of fibroblasts by extrusion into the extracellular matrix in close association with a dedicated receptor, CD44. Fibroblasts also elaborate hyaluronidase, the degradation enzyme, and are able to internalize both HA and, importantly, its breakdown products [5]. Enzymatic degradation cleaves the HA macromolecule into smaller polymers, each comprised of variable lengths of dimeric chains; many of which appear to modulate wound healing even if studies have indicated that most of the effects attributed to the molecule are applicable to only few products. Collagen deposition by fibroblasts is one of the key factors in reconstituting a supporting matrix at sites of scar formation and it is the nature of this deposition that largely determines scar quality. There is evidence that extracellular matrix (ECM) remodeling following HA application is enhanced and collagen deposition more

* Corresponding author at: Department of Clinical and Experimental Medicine, Section of Pharmacology, Torre Biologica 5th Floor, Via Consolare Valeria Gazzi, 98125 Messina, Italy. Tel.: +39 0902213648; fax: +39 0902213300.
E-mail address: Francesco.Squadrito@unime.it (F. Squadrito).
[1] Equally contributed to this paper.

0925-4439/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbadis.2011.03.012

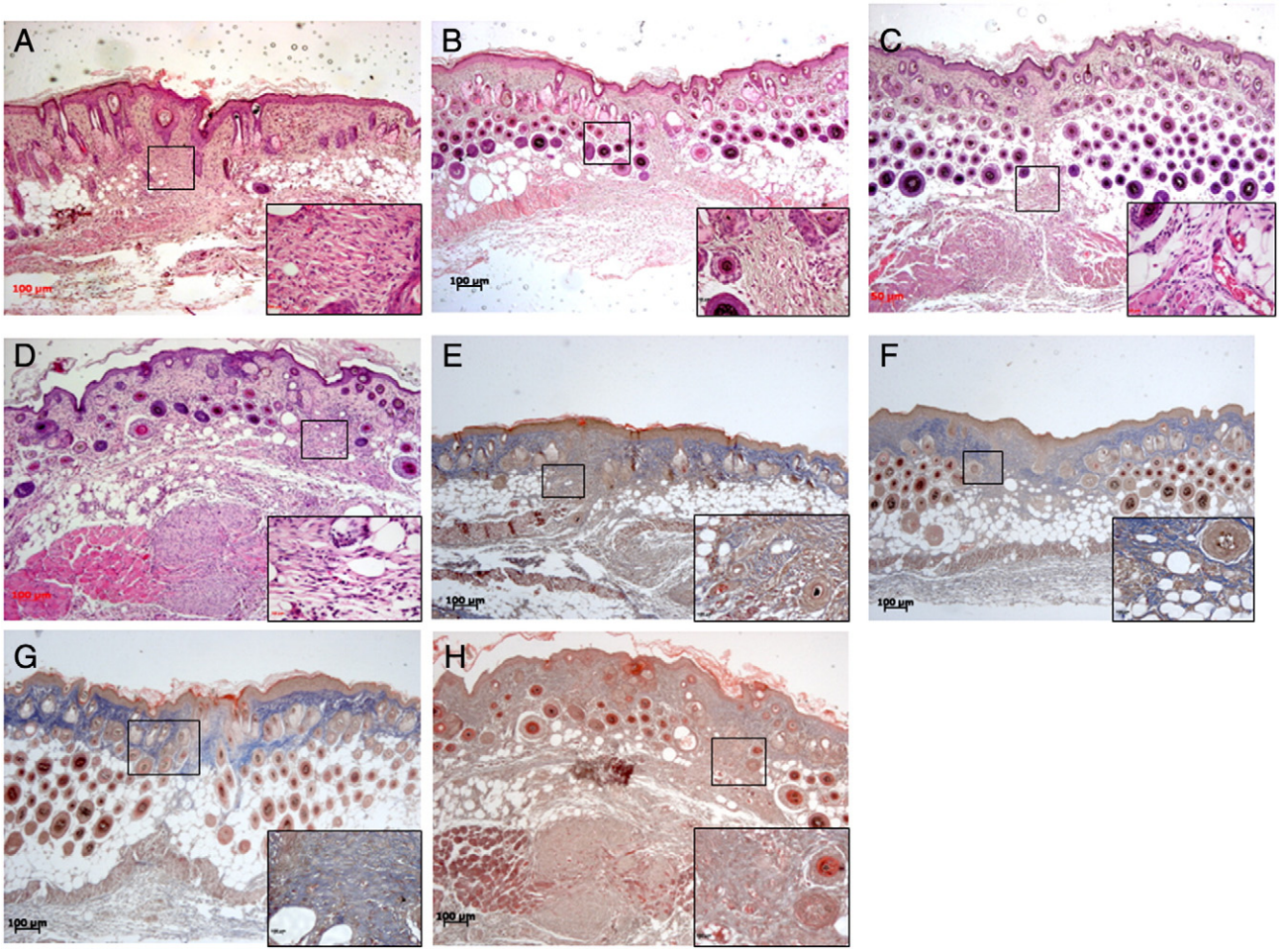

Fig. 1. A–D: Haematoxylin–eosin staining at day 12, original magnification ×10. Rectangle represents the area at higher magnification (×40) in the lower left corner. A: NDB + vehicle, shows almost complete re-epithelialization, presence of granulation tissue and inflammatory infiltrate (see rectangle in the corner). B: NDB + HA 7.5 mg/kg, shows advanced healing with initial retraction of the granulation tissue, less inflammatory infiltrate and presence of hair follicles (see rectangle in the corner). C: NDB + HA 15 mg/kg, shows complete healing process with retraction of the granulation tissue, more mature hair follicles and presence of vessels in the site of incision (see rectangle in the corner). D: NDB + HA 30 mg/kg, shows no further improvement in the healing process compared to NDB + HA 15 mg/kg. E–H: Masson's tricrhome staining at day 12, original magnification ×10. Rectangle represents the area at higher magnification (×40) in the lower left corner. Blue color identifies collagen tissue, red color identifies keratin and muscle fibers. E: NDB + vehicle, shows presence of few and poorly arranged collagen bundles at the incision site (see rectangle in the corner). F: NDB + HA 7.5 mg/kg, shows more and better arranged collagen fibers at the incision site (see rectangle in the corner). G: NDB + HA 15 mg/kg, shows completely restored and mature collagen tissue at the incision site (see rectangle in the corner). H: NDB + HA 30 mg/kg, shows no improvement in collagen tissue (see rectangle in the corner).

ordered [6–8]. Paradoxically, hyaluronidase (which it would be expected to increase tissue HA fragments) causes enhanced scarring [9], whilst persistently raised levels of HA decrease fibroblast contraction [10]. Furthermore, HA fibroblast production may be affected by a number of growth factors [11] and HA degradation products are pro-angiogenic, this effect being limited to fragments of between 4 and 25 disaccharides in length [12].

The mechanism underlying HA degradation is not completely understood, but according to the current model, it involves the concerted action of the somatic hyaluronidases (Hyal-2 and Hyal-1) with CD44 [13]. Very recently it was also shown that Hyal-1 and -2 mRNAs are up-regulated by exogenous lactate in normal fibroblasts [14]. After an injury, during the course of wound repair, the level of oxygen availability decreases whereas lactate production increases. This in turn leads to increased HA turnover and appearance of HA with smaller molecular weight; furthermore it helps to drive adequate wound healing. If the regulation of HA catabolism by lactate is impaired, as it has been shown in fibroblasts from diabetic patients with ulcers [15], tissue injury may promote accumulation of high molecular weight HA and intensification of reduced fibroblast proliferation. This effect may induce transformation of acute wounds into chronic ulcers. Thus, it is possible that both increased accumulation of high molecular weight HA and the elevated level of CD44 expression may be considered as additional factors enhancing susceptibility to chronic ulceration in diabetes.

In addition, HA binding to its cell surface receptor RHAMM is critical for the progression of cells through the cell cycle. The phases of cell cycle are controlled by different types of cyclins in a timely and orderly manner. Cyclins, cyclins-dependent kinases (cdks) and negative regulators such as cyclin-dependent kinase inhibitors work together to regulate cell cycle progression [16].

The genetically diabetic mouse represents an useful animal model for skin healing studies; in fact wound healing in these animals is markedly delayed when compared with non diabetic littermates. Healing impairment is characterized by delayed cellular infiltration, impaired granulation tissue formation, reduced angiogenesis, and decreased collagen synthesis and organization [17–19].

Our group already tested several approaches to improve wound healing by using an incisional wound model in genetically diabetic mice [20–24]. In the present study we investigated whether a systemic injection of HA might also exert beneficial effect on impaired cell cycle and tissue remodeling in diabetic wound healing.

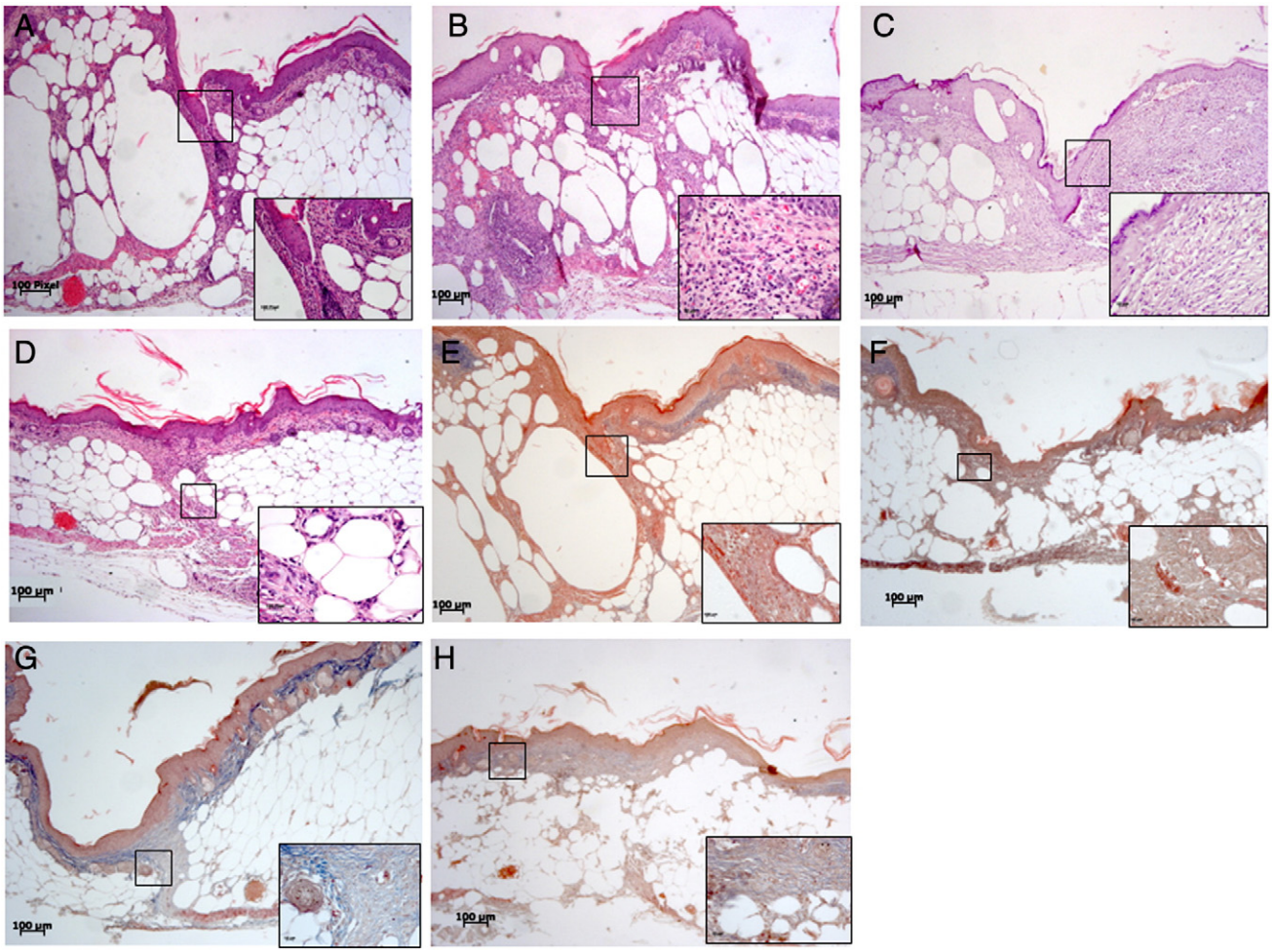

Fig. 2. A–D: Haematoxylin–eosin staining at day 12, original magnification ×10. Rectangle represents the area at higher magnification (×40) in the lower left corner. A: DB + vehicle, shows incomplete re-epithelialization, presence of immature granulation tissue and inflammatory infiltrate (see rectangle in the corner). B: DB + HA 7.5 mg/kg, shows still incomplete healing with reduced inflammatory infiltrate and immature granulation tissue (see rectangle in the corner). C: DB + HA 15 mg/kg, shows partially complete healing process with initial retraction of the granulation tissue (see rectangle in the corner). D: NDB + HA 30 mg/kg, shows further improvement in the healing process, retraction of the granulation tissue (see rectangle in the corner) and presence of hair follicles (see arrows). E–H: Masson's tricrhome staining at day 12, original magnification ×10. Rectangle represents the area at higher magnification (×40) in the lower left corner. Blue color identifies collagen tissue, red color identifies keratin and muscle fibers. E: DB + vehicle, shows presence of very few collagen bundles at the incision site (see rectangle in the corner). F: DB + HA 7.5 mg/kg, shows few collagen fibers at the incision site (see rectangle in the corner). G: DB + HA 15 mg/kg, shows immature collagen tissue at the incision site (see rectangle in the corner). H: DB + HA 30 mg/kg, shows better organized collagen tissue (see rectangle in the corner).

2. Materials and methods

2.1. Animals and experimental protocol

All animal procedures were as humane as possible and complied with the declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals.

Genetically diabetic female C57BL/KsJ-m+/+*Lept*^{db} mice (DB) and their normal littermates (NDB) were obtained from Jackson Laboratory (Bar Harbor, USA). Animals were 10 weeks old at the start of the experiments. Diabetic mice were obese, weighing 30–35 g, while non-diabetic littermates weighed 22–25 g. During the experiments the animals were housed one per cage, maintained under controlled environmental conditions (12 hour light/dark cycle, temperature approximately 23 °C) and provided with standard laboratory food and water ad libitum.

The animals ($n = 56$) were divided into 8 groups of 7 animals each. After general anesthesia with sodium pentobarbital (80 mg/kg/i.p.), hair on the back was shaved and skin washed with povidone–iodine solution and wiped with sterile water.

Two full-thickness longitudinal incisions (4 cm) were made on the dorsum of the mice and the wound edges were closed with 4–0 silk surgical suture placed at 1-cm intervals. Normoglycemic and diabetic mice received either high molecular weight HA (4000 kDa; Sigma-Aldrich, Italy) at different doses (7, 15 and 30 mg/kg/i.p. daily) or its vehicle (100 µl/i.p. of a 0.9% NaCl solution) for 12 days. The animals were sacrificed after 12 days and the wounds removed by using a scalpel to cut the shape of an ellipse around the lesion.

2.2. Histologic examination

Skin specimens were fixed in 10% neutral buffered formalin; after fixation, perpendicular sections of the anterior-posterior axis of the wound were embedded in paraffin. Five micrometer thick sections were mounted on glass slides, de-waxed, rehydrated to distilled water and stained with haematoxylin and eosin or Masson's trichrome according to routine procedures for light microscopy. As part of the histological evaluation, all slides were examined by a pathologist without knowledge of the previous treatment, by means of an eye-piece grid under the microscope from ×20 to ×100 magnification. The following parameters were evaluated: re-epithelialization, dermal matrix deposition and regeneration, granulation tissue formation and remodeling. The histological parameters adopted in this study were evaluated

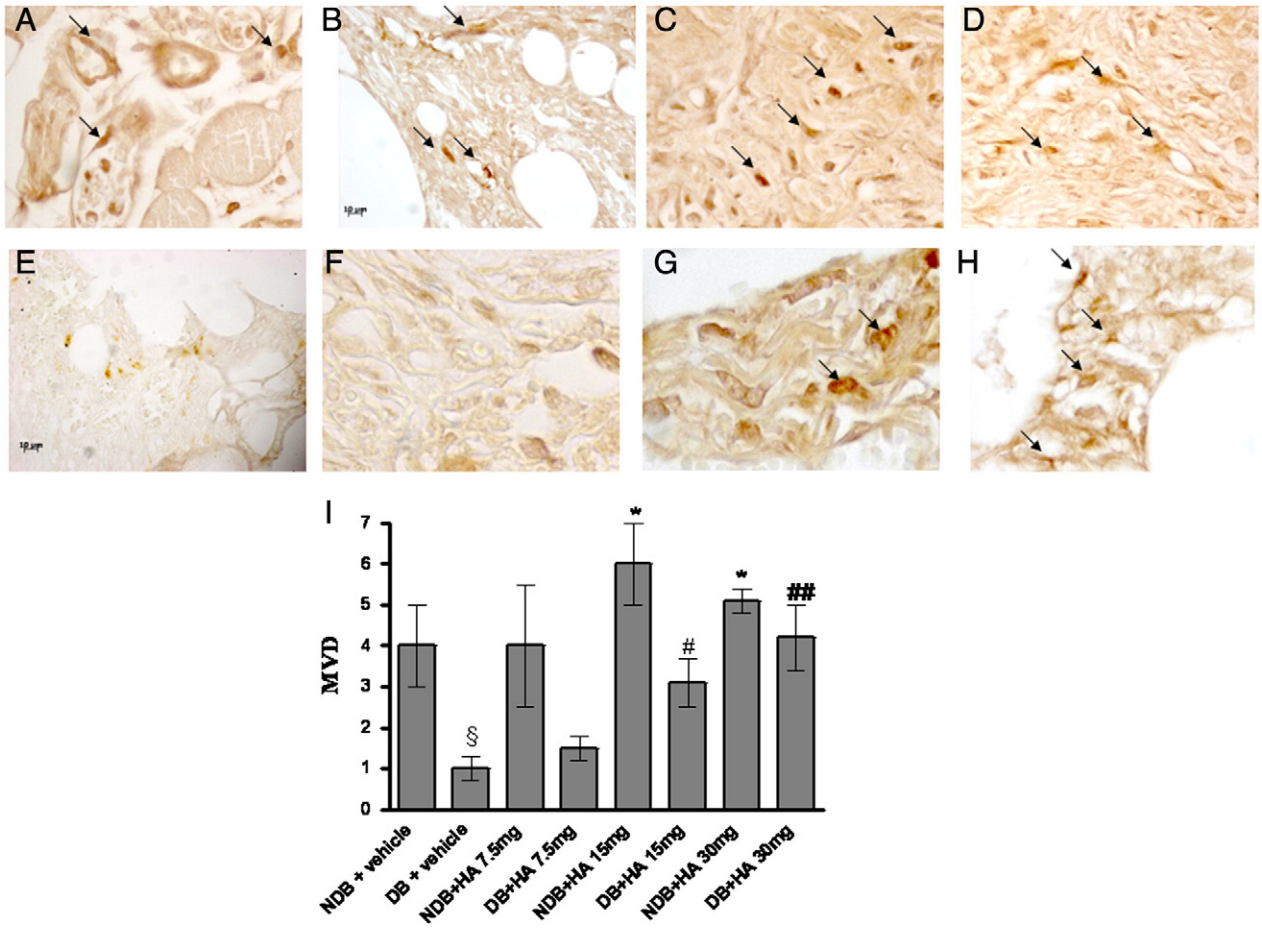

Fig. 3. CD31 immunostaining at day 12, original magnification ×40. Arrows indicate positive staining in small vessels in dermal tissue. A: NDB + vehicle. B: NDB + HA 7.5 mg/kg. C: NDB + HA 15 mg/kg. D: NDB + HA 30 mg/kg. E: DB + vehicle. F: DB + HA 7.5 mg/kg. G: DB + HA 15 mg/kg. H: DB + HA 30 mg/kg. I: The bar graph shows microvascular density quantification carried out by a trained pathologist blinded to treatment, as better specified in the methods. §$p<0.005$ vs. NDB + vehicle; *$p<0.05$ vs. NDB + vehicle; #$p<0.05$ vs. DB + vehicle; ##$p<0.01$ vs. DB + vehicle.

according to the literature data concerning wound healing in experimental models [25].

2.3. Immunohistochemistry

Paraffin embedded tissues were sectioned (5 μ) and antigen retrieval was performed using citrate buffer from BioGenex (San Ramon, CA, USA). Tissues were treated with primary antibody against CD31 (Santa Cruz, USA). Secondary antibody was provided in a kit by Innovex (Richmond, CA, USA) and the location of the reaction was visualized with 3,3′diaminobenzidine tetrahydrochloride (Sigma, Milano, Italy). To assess microvessel density (MVD) three to six areas with the highest staining per section were selected and the number of blood vessels having a visible lumen was counted (×40 magnification) by two pathologists blinded to the samples.

2.4. Tissue hyaluronan sample preparation and HA quantification

At the end of the experiment skin from the wound site was immediately removed and frozen in liquid nitrogen for HA determination. Weighed amounts of frozen tissue (50 mg) were homogenized in 1.0 ml of 150 mM Tris–HCl, pH 8.3; 150 mM sodium chloride; 150 mM calcium chloride; 5 mM deferoxamine and 40 IU of protease K using an Ultra-Turrax homogenizer. The sample was digested overnight at 55 °C and the homogenates clarified by centrifugation at 4 °C (18,000*g* for 5 min). Supernatants were collected and placed in a boiling bath for 20 min to inactivate any proteases.

The assay was carried out using a specific enzyme-linked binding protein assay test kit (cat no. 029-001, Corgenix, Cambridgeshire, UK). In brief, the assay uses microwells coated with a highly specific hyaluronic acid binding protein (HABP) from bovine cartilage to capture HA, and an enzyme-conjugated version of HABP to detect and measure HA. The concentration of HA in each sample was determined by interpolation from a standard curve ranging from 0 to 800 ng/ml.

2.5. Western blot analysis

Skin samples were homogenized in lysis buffer as previously described 22–24 and after protein determination samples were denatured in reducing buffer (62 mM Tris pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.003% bromophenol blue) and separated by electrophoresis on an SDS (12%) polyacrylamide gel. The separated proteins were transferred on to a nitrocellulose membrane using the transfer buffer (39 mM glycine, 48 mM Tris, pH 8.3, 20% methanol) at 200 mA for 1 h. The membranes were stained with Ponceau's (0.005% in 1% acetic acid) to confirm equal amounts of protein and were blocked with 5% non fat dry milk in TBS-0.1% Tween for 1 h at room temperature, washed three times for 10 min each in TBS-0.1% Tween, and incubated with a primary antibody for TGF-β1, transglutaminase-II, cyclin B1, Cdc2 or p27 (Cell Signaling, Beverly, MA) in TBS-0.1% Tween overnight at 4 °C. After being washed three times for 10 min each in TBS-0.1% Tween, the membranes were incubated with a secondary peroxidase-conjugated antibody (Pierce, Rockford, IL) for 1 h at room temperature. After washing, the membranes were analyzed by the enhanced chemiluminescence's

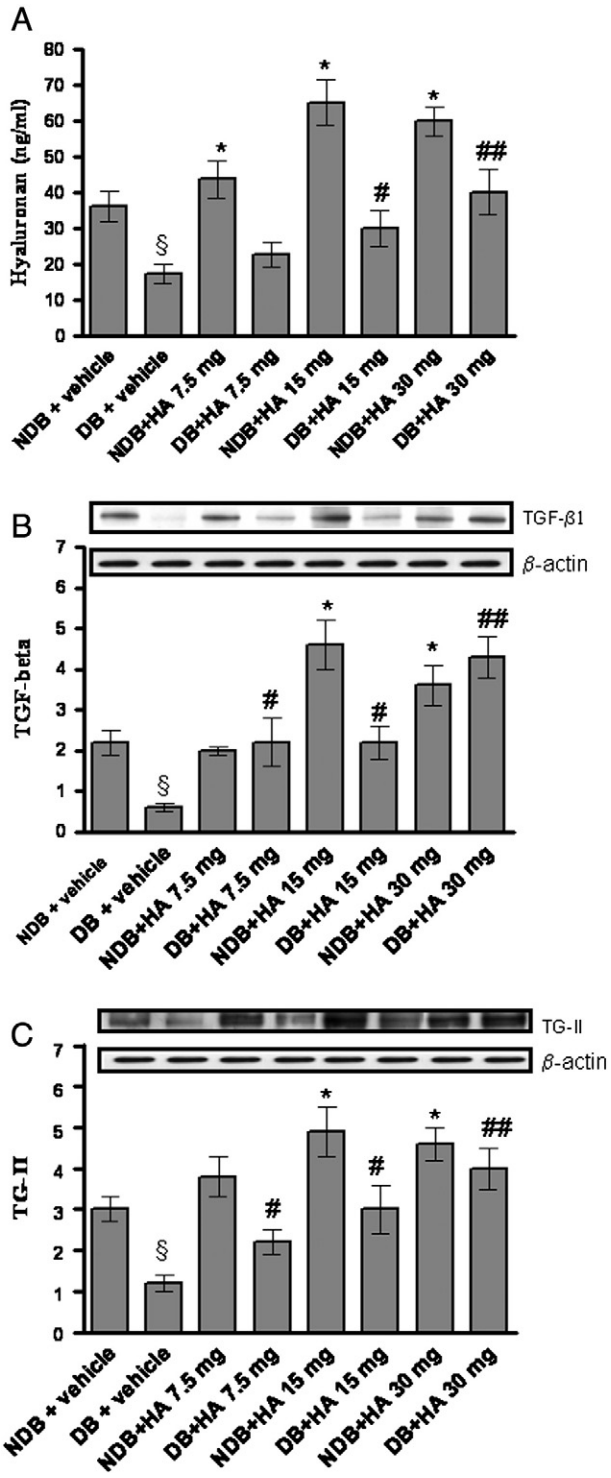

Fig. 4. A: Hyaluronan skin content following 12 days of systemic administration. §$p<0.005$ vs. NDB + vehicle; *$p<0.05$ vs. NDB + vehicle; #$p<0.05$ vs. DB + vehicle; ##$p<0.01$ vs. DB + vehicle. B: Representative Western blot of TGF-β expression in skin samples. §$p<0.005$ vs. NDB + vehicle; *$p<0.05$ vs. NDB + vehicle; #$p<0.05$ vs. DB + vehicle; ##$p<0.01$ vs. DB + vehicle. C: Representative Western blot of transglutaminase-II expression in skin samples. §$p<0.005$ vs. NDB + vehicle; *$p<0.05$ vs. NDB + vehicle; #$p<0.05$ vs. DB + vehicle; ##$p<0.01$ vs. DB + vehicle.

system according to the manufacture's protocol (Amersham, Little Chalfont, UK). The TGF-β, TG-II, cyclin B1, Cdc2 and p27 protein signals were quantified by scanning densitometry using a bio-image analysis system (Bio-Profil Celbio, Milan, Italy). Equal loading of protein was assessed on stripped blots by immunodetection of β-actin with a rabbit monoclonal antibody (Cell Signaling) and a secondary peroxidase-conjugated antibody (Pierce). All antibodies are purified by protein A and peptide affinity chromatography.

2.5.1. Breaking strength

The maximum load (breaking strength) tolerated by wounds was measured blindly on coded samples using a calibrated tensometer (Sans, Milan, Italy) as described previously [24]. The ends of the skin strip were pulled at a constant speed (20 cm min-1), and breaking strength was expressed as the mean maximum level of tensile strength in Newton (N) before separation of wounds.

2.6. Statistical analysis

All data are expressed as the mean plus or minus the standard deviation (mean ± SD). Comparisons between different treatments were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. In all cases, a probability error of less than 0.05 was selected as the criterion for statistical significance. Graphs were drawn using GraphPad Prism (version 4.0 for Windows).

3. Results

3.1. Effects of systemic HA on skin healing and angiogenesis

Both vehicle and HA (at the doses of 7.5 and 15 mg/kg) treated NDB mice showed at day 12 a complete skin repair (Fig. 1A, B and C) with a well formed granulation and collagen tissue (Fig. 1E, F and G). The 30 mg/kg dose of HA was associated with dermal scar formation in NDB mice (Fig. 1D and H). Diabetic wounds from animals administered with vehicle showed at day 12 incomplete re-epithelialization, with a very low organized granulation tissue (Fig. 2A and E). Increasing doses of HA (7.5 and 15 mg/kg) improved the healing process in DB mice (Fig. 2B and C), ameliorating the formation and maturation of granulation tissue and collagen bundles (Fig. 2F and G). Complete re-epithelialization and well formed granulation tissue rich in fibroblasts and collagen were observed in diabetic wounds of mice treated with the 30 mg/kg dose of HA (Fig. 2D and H).

To confirm the angiogenic response, CD31 was investigated by immunostaining. The semi-quantitative assessment of microvascular density showed overlapping values in NDB mice treated with either vehicle (Fig. 3A and I) or HA (at 7.5, 15 and 30 mg/kg; Fig. 3B, C, D and I). By contrast low occurrence of newly formed vessels was observed in samples obtained from DB animals receiving vehicle (Fig. 3E and I) when compared with NDB animals. The administration of increasing doses of HA resulted in a marked enhancement in CD31 staining, especially at the dose of 30 mg/kg (Fig. 3F, G and H), and with a higher concentration of newly formed vessels (Fig. 3I).

3.2. Skin content of hyaluronan after systemic administration

Intact skin mean hyaluronan level was 68.7 ± 5.3 ng/ml in NDB mice and 43.8 ± 4.5 ng/ml in DB mice, thus suggesting a marked impairment in hyaluronan content during diabetes. At day 12, hyaluronan levels in wounds from vehicle-treated NDB mice were higher (36.2 ± 4.2 ng/ml) than in vehicle-treated DB mice (17.3 ± 2.7 ng/ml, Fig. 4A). Systemic administration of HA increased hyaluronan tissue concentration reaching a maximum effect in NDB mice at the dose of 15 mg/kg and in DB mice at the dose of 30 mg/kg, respectively (Fig. 4A).

3.3. Effects of systemic HA on molecular markers of tissue remodeling

The healing process stimulated TGF-β1 expression in NDB mice administered with vehicle; this expression was not augmented in vehicle-treated DB mice (Fig. 4B). HA administration, especially at the dose of 15 mg/kg, markedly enhanced TGF-β1 expression in NDB mice

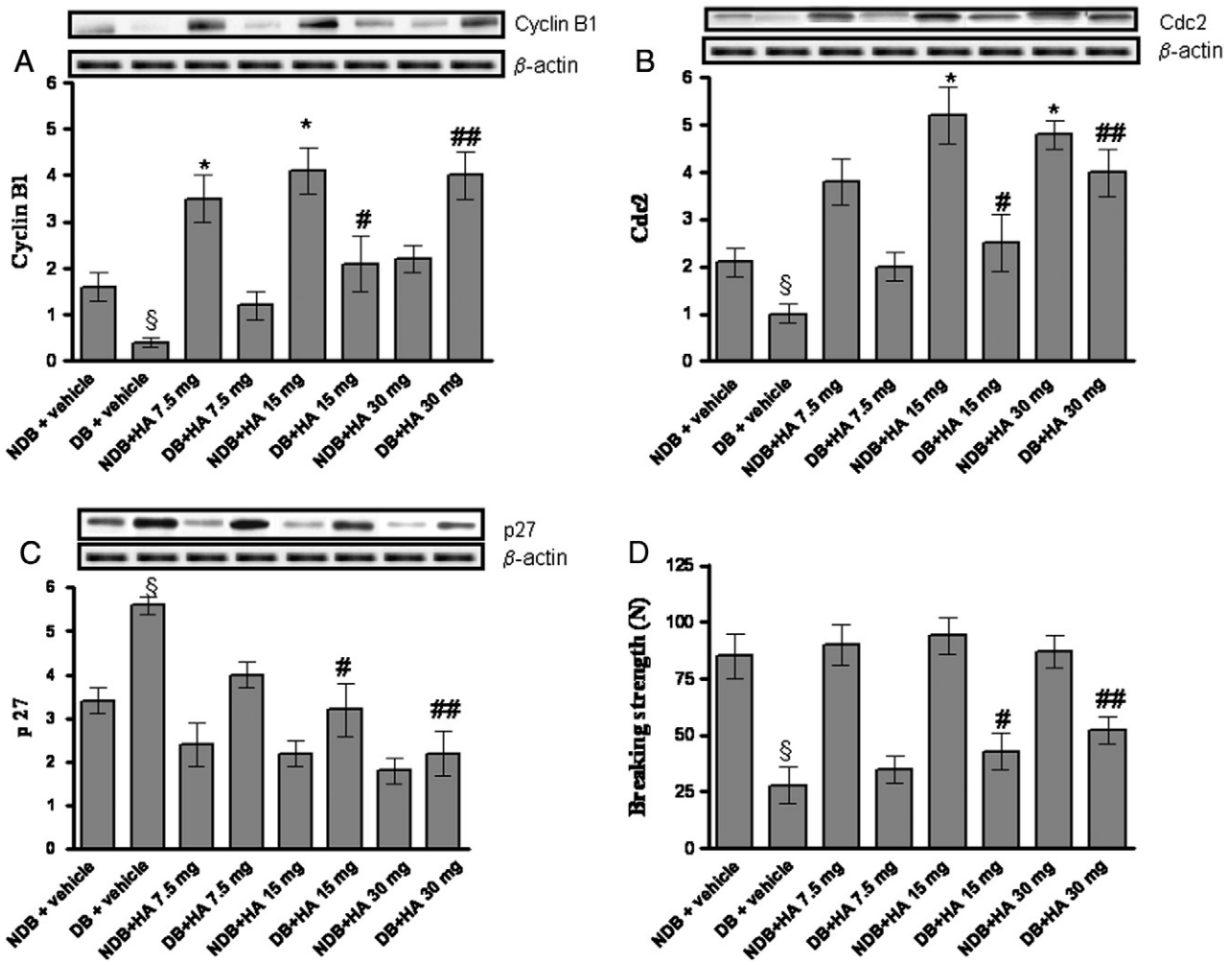

Fig. 5. A: Representative Western blot of cyclin B1 expression in skin samples. §$p<0.005$ vs. NDB + vehicle; *$p<0.05$ vs. NDB + vehicle; #$p<0.05$ vs. DB + vehicle; ##$p<0.01$ vs. DB + vehicle. B: Representative Western blot of Cdc2 expression in skin samples. §$p<0.005$ vs. NDB + vehicle; *$p<0.05$ vs. NDB + vehicle; #$p<0.05$ vs. DB + vehicle; ##$p<0.01$ vs. DB + vehicle. C: Representative Western blot of p27 expression in skin samples. §$p<0.005$ vs. NDB + vehicle; #$p<0.05$ vs. DB + vehicle; ##$p<0.01$ vs. DB + vehicle. D: Breaking strength of skin samples following 12 days of systemic administration. §$p<0.005$ vs. NDB + vehicle; #$p<0.05$ vs. DB + vehicle; ##$p<0.01$ vs. DB + vehicle.

(Fig. 4B). Samples obtained from DB mice treated with increasing doses of HA showed a significant enhancement in TGF-β1 expression, being the 30 mg/kg dose the most effective one (Fig. 4B). DB mice treated with vehicle had a lower expression of TG-2 than vehicle-treated NDB (Fig. 4C). Increasing doses of HA augmented TG-2 expression both in NDB and DB mice; the maximum effect was observed with the 15 mg/kg dose in NDB and with the 30 mg/kg dose in DB mice, respectively (Fig. 4C).

3.4. Effects of systemic HA on cell cycle regulators

The cell cycle positive regulators cyclin B1 and its kinase Cdc2, both specifically activated during the mitotic phase of the cell cycle, were evaluated at day 12 post-wounding. NDB animals showed a robust expression of the two positive regulators; cyclin B1 and Cdc2 were significantly up-regulated by HA, being the 15 mg/kg the most effective dose (Fig. 5A and B). DB animals treated with vehicle had a lower expression of cyclin B1 (Fig. 5A) and Cdc2 (Fig. 5B) than NDB. Cyclin B1 and Cdc2 expression was significantly increased by HA and the 30 mg/kg dose produced the maximum effect (Fig. 5A and B).

The cell cycle negative regulator p27 was significantly higher at day 12 in DB mice than in NDB, thus suggesting an altered regeneration process during diabetes (Fig. 5C). Systemic increasing doses of HA (with a maximum effect observed at 30 mg/kg) succeeded in reducing p27 expression in DB animals (Fig. 5C). By contrast HA did not modify the negative regulator in NDB animals (Fig. 5C).

3.4. Effects of systemic HA on tensile strength of diabetic wounds

Wound breaking strength for each group at day 12 is shown in Fig. 5D. Wound breaking strength of DB mice was significantly lower than that of NDB animals ($p<0.001$). HA administration did not further increase the tensile strength of the wounds in NDB. By contrast, breaking strength of incisional diabetic wounds was improved by HA treatment being the 30 mg/kg the most effective dose.

4. Discussion

HA is a major extracellular glycosaminoglycan and is found in most types of extracellular matrices where it provides primarily a structural role. The highest HA concentrations are found in typical connective tissues such as umbilical cord, synovial fluid of joints and the vitreous body of the eye [26–28]. In marked contrast with all other glycosaminoglycans (GAGs) HA is not synthesized in the Golgi apparatus, but on the cytoplasmic surface of the plasma membrane [29]. HA is generally produced in the mesenchymal connective tissue of the body. In animals HA is formed at the cell surface of fibroblasts by extrusion into the extracellular matrix in close association with a dedicated receptor,

CD44. As well as manufacturing the macromolecule, fibroblasts also elaborate hyaluronidase [30], the enzyme responsible for its degradation, and are able to internalize both the original molecule and its breakdown products [31]. HA production increases during tissue remodeling and has been already associated with the wound repair process [32–35]. After tissue injury, HA fragments of lower molecular mass accumulate, as a result of excessive digestion by hyaluranidase. Generation of HA fragments under conditions of tissue injury may signal to the host that normal homeostasis has been profoundly disturbed. Despite their simple primary structure HA fragments have wide-ranging functions in wound healing that are size-specific [36]. It has been shown that in human chronic dermal wounds and in intact and wounded skin of diabetic rats the amount of extracellular matrix components, in particular HA, is drastically decreased as a result of a reduced rate of their biosynthesis, increased degradation or a combination of both phenomena [37,38]. Accordingly, HA content was very low and the healing process was markedly delayed (in terms of re-epithelialization, collagen tissue formation and angiogenesis) in our diabetic vehicle-treated mice. The systemic administration of HA resulted in a significant improvement in scar formation, collagen synthesis and deposition and formation of new vessels at the wound site. Furthermore, the Masson's trichrome stain showed a stimulating action of HA on fibroblasts with augmented granulation tissue formation, as also confirmed by the enhanced expression of TGF-β1 and TG-II; this latter has been identified as a "natural glue" in the intercellular matrix that plays a key role in all restorative processes occurring during wound healing. TGF-β1 can present contradictory effects on skin, but it is well known that this mediator, released by cells that are localized at sites of tissue repair, such as platelets, activated macrophages and possibly fibroblasts, exerts beneficial effects in each phase of wound healing [39,40]. TGF-β1 is necessary in the inflammatory phase for proper chemoattraction of monocytes; in the proliferative phase, it stimulates extracellular matrix production and affects angiogenesis and epithelialization, and, moreover, induces myofibroblast contraction and formation in skin wounds. Recently, TGF-β1 increased the rate of healing and the breaking strength of the repaired tissue in aged animals; it also enhanced angiogenesis and consequent blood flow to dermal wounds in these animals, by stimulating, at least in part, local release of other growth factors [41].

HA fragments are believed to stimulate cell cycle progression; this activity seems to be initiated through its binding to both the cell surface receptor CD44 and the receptor for HA-mediated motility (RHAMM), resulting in signal transduction activation and ultimately mitogenesis [42,43]. The mechanism for the HA induced promotion of cells through the cell cycle may be identified in the HA–RHAMM signaling that stimulates Cdc2/cyclin B1 complex kinase activity [43]. Previous data from our laboratory showed that diabetic animals have an impaired cell cycle machinery that may explain the delayed healing process [44]. Here we show an increased expression of the specific cyclin B1/Cdc2 kinase complex together with a concomitant reduction in the negative regulator p27 following 12 days of systemic HA administration. This led us to hypothesize that HA beneficial effects in the disturbed skin repair process of diabetes may be mainly ascribed to its ability to restore the impaired cell cycle machinery.

Diabetic animals had lower basal content of HA than normoglycemic mice. This might explain why the most effective dose of HA was different between the two strains of mice The highest dose of HA produced the best results in diabetics, improving significantly the wound healing process and augmenting skin mechanical properties. On the contrary administration of the highest dose of HA caused dermal scar formation (fibrosis) in normoglycemic mice. The explanation for this discrepancy may stem in the normal HA turnover of non diabetic animals. The size of HA, in fact, influences its biological activity; small HA oligosaccharides stimulate angiogenesis and fibroblast proliferation, whereas high levels of macromolecular HA inhibit angiogenesis and promote fibrosis. Therefore it is tempting to speculate that the highest dose of HA is not broken down in normoglycemic animals because the balance between formation and degradation of the ECM is in "equilibrium"; this would cause a lack of HA degradation and a consequent excessive occurrence of macromolecular HA that would trigger fibrosis. To date this is the first report showing that systemic administration of HA in diabetic animals induces an improvement in the healing process together with a stimulation of the cell cycle machinery. In addition we previously reported that systemic high-molecular weight HA injection may also reduce diabetes-induced renal injury [45]. These findings, taken together, are of particular importance in a clinical setting; in fact, they suggest that systemic administration of HA may counteract nephropathy and ulcers, two of the most severe complication of diabetes.

5. Conclusion

Diabetic ulcers and nephropathy are main causes of morbidity and mortality in diabetic patients. Systemic HA might be a potential therapeutic strategy to manage these two diabetic complications that share, as a common pathogenesis, alterations in the extracellular matrix turnover.

References

[1] V. Falanga, Wound healing and its impairment in the diabetic foot, Lancet 366 (2005) 1736–1743.
[2] N. Singh, D.G. Armstrong, B.A. Lipsky, Preventing foot ulcers in patients with diabetes, JAMA 293 (2005) 217–228.
[3] G.E. Reiber, G.J. Raugi, Preventing foot ulcers and amputations in diabetes, Lancet 366 (2005) 1676–1677.
[4] P.H. Weigel, S.J. Frost, R.D. LeBoeuf, C.T. McGary, The specific interaction between fibrin(ogen) and hyaluronan: possible consequences in haemostasis, inflammation and wound healing, Ciba Found. Symp. 143 (1989) 248–261.
[5] Y. Kobayashi, A. Okamoto, K. Nishinari, Viscoelasticity of hyaluronic acid with different molecular weights, Biorheology 31 (1994) 235–244.
[6] J.A. Iocono, T.M. Krummel, K.A. Keefer, G.M. Allison, H. Paul, Repeated additions of hyaluronan alters granulation tissue deposition in sponge implants in mice, Wound Repair Regen. 6 (1998) 442–448.
[7] C.M. Kielty, S.P. Whittaker, M.E. Grant, C.A. Shuttleworth, Type IV collagen microfibrils: evidence for a structural association with hyaluronan, J. Cell Biol. 118 (1992) 979–990.
[8] P. Rooney, S. Kumar, Inverse relationship between hyaluronan and collagens in development and angiogenesis, Differentiation 54 (1993) 1–9.
[9] D.C. West, D.M. Shaw, P. Lorenz, N.S. Adzick, M.T. Longaker, Fibrotic healing of adult and late gestation fetal wounds correlates with increased hyaluronidase activity and removal of hyaluronan, Int. J. Biochem. Cell Biol. 29 (1997) 201–210.
[10] L.L. Huang-Lee, M.E. Nimni, Fibroblast contraction of collagen matrices with and without covalently bound hyaluronan, J. Biomater. Sci. Polym. Ed. 5 (1993) 99–109.
[11] P. Heldin, T.C. Laurent, C.H. Heldin, Effect of growth factors on hyaluronan synthesis in cultured human fibroblasts, Biochem. J. 258 (1989) 919–922.
[12] D.C. West, I.N. Hampson, F. Arnold, S. Kumar, Angiogenesis induced by degradation products of hyaluronic acid, Science 228 (1991) 1324–1326.
[13] G. Lepperdinger, J. Mullegger, G. Kreil, Hyal2—less active, but more versatile? Matrix Biol. 20 (2001) 509–514.
[14] B. Formby, R. Stern, Lactate-sensitive response elements in genes involved in hyaluronan catabolism, Biochem. Biophys. Res. Commun. 305 (2003) 203–208.
[15] D.L. Coleman, Diabetes-obesity syndromes in mice, Diabetes 31 (1982) 1–6.
[16] T.K. MacLachlan, N. Sang, A. Giordano, Cyclins, cyclin-dependent kinases and cdk inhibitors: implications in cell cycle control and cancer, Crit. Rev. Eukaryot. Gene Expr. 5 (1995) 127–156.
[17] H.G. Bohlen, B.A. Niggl, Adult microvascular disturbances as a result of juvenile-onset diabetes in db/db mice, Blood Vessels 16 (1979) 269–276.
[18] W.H. Goodson, T.K. Hunt, Studies of wound healing in experimental diabetes, J. Surg. Res. 22 (1977) 221–227.
[19] N.Y. Yevdokimova, S.E. Podpryatov, Hyaluronic acid production and CD44 expression in cultured dermal fibroblasts of patients with non-insulin-dependent diabetes mellitus with and without chronic ulcers on the lower extremity, Wound Repair Regen. 13 (2005) 181–188.
[20] D. Altavilla, A. Saitta, D. Cucinotta, M. Galeano, B. Deodato, M. Colonna, V. Torre, G. Russo, A. Sardella, G. Urna, G.M. Campo, V. Cavallari, G. Squadrito, F. Squadrito, Inhibition of lipid peroxidation restores impaired vascular endothelial growth factor expression and stimulates wound healing and angiogenesis in the genetically diabetic mouse, Diabetes 50 (2001) 667–674.
[21] A. Bitto, L. Minutoli, D. Altavilla, F. Polito, T. Fiumara, H. Marini, M. Galeano, M. Calò, P. Lo Cascio, M. Bonaiuto, A. Migliorato, A.P. Caputi, F. Squadrito, Simvastatin enhances VEGF production and ameliorates impaired wound healing in experimental diabetes, Pharmacol. Res. 57 (2008) 159–169.
[22] A. Bitto, L. Minutoli, M.R. Galeano, D. Altavilla, F. Polito, T. Fiumara, M. Calò, P. Lo Cascio, L. Zentilin, M. Giacca, F. Squadrito, Angiopoietin-1 gene transfer improves impaired wound healing in genetically diabetic mice without increasing VEGF expression, Clin. Sci. (Lond) 114 (2008) 707–718.

[23] M. Galeano, D. Altavilla, D. Cucinotta, G.T. Russo, M. Calò, A. Bitto, H. Marini, R. Marini, E.B. Adamo, P. Seminara, L. Minutoli, V. Torre, F. Squadrito, Recombinant human erythropoietin stimulates angiogenesis and wound healing in the genetically diabetic mouse, Diabetes 53 (2004) 2509–2517.
[24] M. Galeano, A. Bitto, D. Altavilla, L. Minutoli, F. Polito, M. Calò, P. Lo Cascio, F. Stagno d'Alcontres, F. Squadrito, Polydeoxyribonucleotide stimulates angiogenesis and wound healing in the genetically diabetic mouse, Wound Repair Regen. 16 (2008) 208–217.
[25] T.A. Dechert, A.E. Ducale, S.I. Ward, D.R. Yager, Hyaluronan in human acute and chronic dermal wounds, Wound Repair Regen. 14 (2006) 252–258.
[26] R.K. Reed, K. Lilja, T.C. Laurent, Hyaluronan in the rat with special reference to the skin, Acta Physiol. Scand. 134 (1988) 405–411.
[27] J.R. Fraser, W.G. Kimpton, B.K. Pierscionek, R.N.P. Cahill, The kinetics of hyaluronan in normal and acutely inflamed synovial joints: observations with experimental arthritis in sheep, Arthritis Rheum. 22 (1993) 9–17.
[28] U.B. Laurent, T.C. Laurent, L.K. Hellsing, L. Persson, M. Hartman, K. Lilja, Hyaluronan in human cerebrospinal fluid, Acta Neurol. Scand. 94 (1996) 194–206.
[29] P. Prehm, Hyaluronate is synthesized in plasma membranes, Biochem. J. 220 (1984) 579–600.
[30] S. Stair, T.B. Csoka, R. Stern, Hyaluronidase expression in human skin fibroblasts, Biochem. Biophys. Res. Commun. 266 (1999) 268–273.
[31] C.N. Bertolami, S. Berg, D.V. Messadi, Binding and internalisation of hyaluronate by human cutaneous fibroblasts, Matrix 12 (1992) 11–21.
[32] R.D. Price, S. Myers, I.M. Leigh, R.D. Price, The role of hyaluronic acid in wound healing, Am. J. Clin. Dermatol. 6 (2005) 393–402.
[33] R.D. Price, M.G. Berry, H.A. Navsaria, Hyaluronic acid: the scientific and clinical evidence, J. Plast. Reconstr. Aesthet. Surg. 60 (2007) 1110–1119.
[34] W.Y. Chen, G. Abatangelo, Functions of hyaluronan in wound repair, Wound Rep. Reg. 7 (1999) 79–89.
[35] R. Stern, H.I. Maibach, Hyaluronan in skin: aspects of aging and its pharmacologic modulation, Clin. Dermatol. 26 (2008) 106–122.
[36] R. Stern, A.A. Asari, K.N. Sugahara, Hyaluronan fragments: an information-rich system, Eur. J. Cell Biol. 85 (2006) 699–715.
[37] M. Cechowska–Pasko, J. Palja, E. Bankowski, Decrease in the glycosaminoglycan content in the skin of diabetic rats. The role of IGF-I, IGF-binding proteins and proteolytic activity, Mol. Cell. Biochem. 154 (1996) 1–8.
[38] M. Cechowska–Pasko, J. Palja, E. Bankowski, Alterations in glycosaminoglycans in wounded skin of diabetic rats. A possible role of IGF-I, IGF-binding proteins and proteolytic activity, Acta Biochim. Pol. 43 (1996) 557–566.
[39] J.P. Grande, Role of transforming growth factor-beta in tissue injury and repair, Proc. Soc. Exp. Biol. Med. 214 (1997) 27–40.
[40] B.R. Klass, A.O. Grobbelaar, K.J. Rolfe, Transforming growth factor beta1 signalling, wound healing and repair: a multifunctional cytokine with clinical implications for wound repair, a delicate balance, Postgrad. Med. J. 85 (2009) 9–14.
[41] D.H. Adams, X.L. Strudwick, Z. Kopecki, J.A. Hooper-Jones, K.I. Matthaei, H.D. Campbell, B.C. Powell, A.J. Cowin, Gender specific effects on the actin remodelling protein flightless I and TGF-beta1 contribute to impaired wound healing in aged skin, Int. J. Biochem. Cell Biol. 40 (2008) 1555–1569.
[42] R.M. Greco, J.A. Iocono, H.P. Ehrlich, Hyaluronic acid stimulates human fibroblast proliferation within a collagen matrix, J. Cell. Physiol. 177 (1998) 465–473.
[43] F. Gao, C.X. Yang, W. Mo, Y.W. Liu, Y.Q. He, Hyaluronan oligosaccharides are potential stimulators to angiogenesis via RHAMM mediated signal pathway in wound healing, Clin. Invest. Med. 31 (2008) E106–E116.
[44] D. Altavilla, F. Squadrito, F. Polito, N. Irrera, M. Calò, P. Lo Cascio, M. Galeano, L. La Cava, L. Minutoli, H. Marini, A. Bitto, Activation of adenosine A2(A) receptors restores the altered cell-cycle machinery during impaired wound healing in genetically diabetic mice, Surgery 149 (2011) 253–261.
[45] G.M. Campo, A. Avenoso, A. Micali, G. Nastasi, F. Squadrito, D. Altavilla, A. Bitto, F. Polito, M.G. Rinaldi, A. Calatroni, A. D'Ascola, S. Campo, High molecular weight hyaluronan reduced renal PKC activation in genetically diabetic mice, Biochim. Biophys. Acta 1802 (2010) 1118–1130.